PROCESSES AND AGENTS FOR DETECTING LISTERIAS

Background of the Invention

The invention relates to agents and processes for detecting bacteria of the genus *Listeria*, in particular *L. monocytogenes*.

The members of the genus *Listeria* are gram-positive rod-shaped bacteria which occur ubiquitously. Seven different species belong to this genus: *L. monocytogenes, L. ivanovii, L. seeligeri, L. welshimeri, L. innocua, L. murrayi* and *L. grayi*. Of these, only *L. monocytogenes* is pathogenic for humans, endangering in particular the newly born, pregnant women and older persons, as well as patients subjected to immunosuppression. *L. monocytogenes* infections frequently have a fatal outcome.

Contaminated foodstuffs, in which the organism can multiply even at low temperatures around 4°C, are frequently the cause of listeria infections. Thus, various listeria epidemics have been attributed to the consumption of contaminated food, such as, for example, raw milk, cheese or coleslaw. Consequently, rapid detection processes for detecting listerias, in particular in foodstuffs or clinical samples, are urgently required. These processes must additionally be able to distinguish between *L. monocytogenes* and the species which are not pathogenic for humans. Furthermore, it must be possible to detect all variants of *L. monocytogenes*, which is the species which is pathogenic for humans. Recent discussions resulted in the proposal to use *L. innocua* as indicator organism for a potential contamination with *L. monocytogenes*. Therefore, the detection of *L. innocua* would be very useful as well.

Detection of *L. monocytogenes* is effected in a known manner using processes which are based on culturing the microorganisms. The process described in Int. J. Food Microbiol. 4 (1987), 249-256 takes two weeks. A somewhat faster process is recommended by the International Dairy Foundation (IDF); however, it takes at least 6-8 days. Both processes are unsuitable for rapid identification because of the time they take. In addition, both processes are labor-intensive, since nutrient media must be inoculated re-

peatedly in order to obtain single colonies, and since the isolates must subsequently be characterized using biochemical and serological methods of investigation.

While the immunological tests which are currently on the market only take a few hours, they do not permit the important differentiation between different species of listerias. In these processes, also, a two-day pre-enrichment cultivation is required.

A method is described in Appl. Environ. Microbiol. 54 (1988), 2933-2937 in which *L. monocytogenes* is specifically detected using synthetic oligodeoxyribonucleotide probes. However, the probes which are used are not sufficiently specific, since they also react with the species *L. seeligeri*, which is not pathogenic for humans. Prior multiplication of the organisms is required for this process as well: samples of foodstuffs, or their dilutions, are spread on agar plates, and then the inoculated plates are incubated and subsequently investigated by the colony hybridization procedure using a radioactively labelled DNA probe. Detection takes place by autoradiography. This method, too, is labor-intensive and time-consuming.

The DNA sequence of the *iap* (invasion-associated protein) gene of *L. monocytogenes* is described in Infect. Immun. 58, 1943-1950 (1990). This gene encodes a protein which is also known under the designation p60 and which occurs in variants in all *Listeria* species. In *L. monocytogenes* this protein is responsible for the ability to invade animal cells. A polynucleotide (400 bases) having a component sequence from this gene is suitable as a DNA probe for distinguishing *L. monocytogenes* from other organisms.

The polymerase chain reaction (PCR) permits the in vitro amplification of nucleic acids, and prior cultivation is generally not necessary when using this process. In order to start the reaction, short nucleic acid fragments (primers) are required, which primers encompass the section of the genome which is to be amplified. Usually, two primers are required, each of which hybridizes with one nucleic acid strand. One of the primers therefore possesses the complementary sequence to the relevant section of the gene. The choice of these primers determines the specificity of the detection reaction. The use of this process for detecting *L. monocytogenes* is described in Appl. Environmental Microbiology 57, 606-609 (1991), in Letters Appl. Microbiol. 11, 158-162 (1990) and in J. Appl. Bact. 70, 372-379 (1991). More extensive information regarding the details of these processes is available in these publications. The DNA primers bind to the gene for listeriolysin, the listeria hemolysin. The specificity of these primers is at least uncertain, as is evident from comments in J. Appl. Bact. 70: *L. seeligeri* cannot be differentiated with certainty from *L. monocytogenes*. The unambiguous detection of *L. monocytogenes* has thus hitherto not been possible using the PCR technique.

Polyclonal antibodies against *L. monocytogenes* p60 also react with the p60 protein of other, non-pathogenic *Listeria* species. Such antibodies are therefore unsuitable for specifically detecting *L. monocytogenes* by immunological processes. It is possible in principle to purify a polyvalent antiserum of this nature by the specific absorption of interfering antibody fractions: for this purpose, p60 protein from all the other *Listeria* species is covalently bound to carriers. The unwanted antibody fractions can be specifically absorbed; an antiserum then remains which only reacts with p60 protein from *L. monocytogenes*. This method for obtaining an *L. monocytogenes*-specific serum is elaborate: substantial quantities of the polyvalent antiserum are required as starting material, as are, in addition, the p60 iap gene products of the different *Listeria* species. The obtention of monoclonal antibodies against p60 protein would not be associated with this large material requirement; nevertheless, the raising of antibodies against particular epitopes depends on chance: it is first of all necessary to prepare a large number of antibody-producing cell clones, from which suitable clones must then be selected. It has thus far not been possible to obtain antibodies in a targeted manner against epitopes which are specific for *L. monocytogenes*. The same holds true for epitopes which are specific for *L. innocua*.

Summary of the Invention

It is the object of the present invention to provide improved agents and methods for differentiating bacteria of the genus *Listeria*, in particular for detecting bacteria of the species *L. monocytogenes*. In particular, primer sequences which are suitable for the PCR technique are provided according to the invention, as are peptides for the targeted production of specific antibodies which are suitable for the immunological detection of the species *L. monocytogenes* and *L. innocua*.

Upon further study of the specification and appended claims, further objects and advantages of this invention will become apparent to those skilled in the art.

The invention relates to primers, selected from the iap gene, for the amplification of nucleic acids, for example by means of the polymerase chain reaction, characterized in that the primers contain, as a component sequence, at least one sequence according to one of the formulae Ia to Ih and/or an affiliated complementary sequence, it being possible for up to 20 further nucleotide moieties to be bound in front of and/or behind this component sequence. Such primers are suitable for detecting and differentiating bacteria of the genus *Listeria*, including in particular the species *L. monocytogenes*, by means of PCR.

AATATGAAAAAAGC	(SEQ ID NO:1)	Ia
GCTTCGGTCGCGTA	(SEQ ID NO:2)	Ib
ACAGCTGGGATTGC	(SEQ ID NO:3)	Ic
ACTGCTAACACAGCT	(SEQ ID NO:4)	Id
TAACAGCAATTCAAG	(SEQ ID NO:5)	Ie
CTGAGGTAGCGAGC	(SEQ ID NO:6)	If
AGCACTCCAGTTGTTA	(SEQ ID NO:7)	Ig
GCAGTTTCTAAACCT	(SEQ ID NO:8)	Ih

In this context, primers are particularly preferred which contain a sequence according to one of the formulae IIa to IIh and/or an affiliated complementary sequence.

A1 ~~GTCGACTGAATATGAAAAAAAGCAAC~~		IIa
A1 TTGGCTTCGGTCGCGTAGAATTCATA		IIb
GCTACAGCTGGGATTGCGGT	(SEQ ID NO:11)	IIc
CAAACTGCTAACACAGCTACT	(SEQ ID NO:12)	IId
CAATAACAGCAATTCAAGTGC	(SEQ ID NO:13)	IIe
TAACTGAGGTAGCGAGCGAA	(SEQ ID NO:14)	IIf
ACTAGCACTCCAGTTGTTAAAC	(SEQ ID NO:15)	IIg
CCAGCAGTTTCTAAACCTGCT	(SEQ ID NO:16)	IIh

The invention additionally relates to peptides which contain, as a component sequence, at least one sequence according to one of the formulae IIIa to IIIi, it being possible for in each case up to seven amino acids to be bound by peptide linkages in front of and/or behind this component sequence.

ProValAlaProThrGln	(SEQ ID NO:17)	IIIa
ThrGlnAlaThrThrProAla	(SEQ ID NO:18)	IIIb
AlaIleLysGlnThrAlaAsnThrAla	(SEQ ID NO:19)	IIIc
GlnGlnThrAlaProLysAlaProThr	(SEQ ID NO:20)	IIId
ValAsnAsnGluValAlaAlaAlaGluLysThrGlu		IIIe (SEQ ID NO:21)
ThrProValValLysGlnGluValLys	(SEQ ID NO:22)	IIIf
ValLysGlnProThrThrGlnGlnThrAlaPro		IIIg (SEQ ID NO:23)
IleLysGlnProThrLysThrValAlaPro		IIIh (SEQ ID NO:24)
GlnGlnThrThrThrLysAlaProThr		IIIi (SEQ ID NO:25)

In this context, peptides are particularly preferred which have a sequence according to one of the Figures 2a-i, and Figures 5a-d.

The invention also relates to the use of one of the said peptides, having a component sequence according to one of the formulae IIIa to IIIi, for preparing immunogenic conjugates. Peptides having a sequence according to one of the Figures 2a-i and of the Figures 5a-d are particularly preferred for this purpose.

The invention also relates to an antibody which binds an epitope which is formed from the polypeptide according to Figure 3 (SEQ ID NO:39) or contains a peptide according to one of the formulae IIIa-IIIi, preferably according to one of the Figures 2a-i and of Figures 5a-d.

The invention further relates to an antibody which can be prepared by immunizing an experimental animal with a polypeptide according to Figure 3 or with an immunogenic conjugate which contains a peptide having 7 to 24 amino acids selected from the polypeptide according to Figure 3.

The invention also relates to a process for preparing an antibody directed against the p60 protein from listerias by immunizing an experimental animal with an immunogen and isolating the antibodies, characterized in that a polypeptide according to Figure 3 or an immunogenic conjugate which contains a polypeptide according to Figure 3 is used as the immunogen. In this context, immunogenic conjugates are preferred which contain a peptide having 7 to 24 amino acids selected from the polypeptide according to Figure 3, or which contain a peptide according to one of the formulae IVa-IVi, in which

X^3 and X^4 are each independently of one another hydrogen, an arbitrary amino acid or an arbitrary oligopeptide having up to 7 amino acids.

X^3ProValAlaProThrGlnX^4 (SEQ ID NO:17) IVa

X^3ThrGlnAlaThrThrProAlaX^4 (SEQ ID NO:18) IVb

X^3AlaIleLysGlnThrAlaAsnThrAlaX^4 (SEQ ID NO:19) IVc

X^3GlnGlnThrAlaProLysAlaProThrX^4 (SEQ ID NO:20) IVd

X^3ValAsnAsnGluValAlaAlaAlaGluLysThrGluX^4 IVe (SEQ ID NO:21)

~~X^3ThrProValLysGlnGluValLysX^4~~ IVf

~~X^3ValLysGlnProThrThrGlnGlnThrAlaProX^4~~ IVg (SEQ ID NO:23)

X^3IleLysGlnProThrLysThrValAlaProX^4 IVh (SEQ ID NO:24)

X^3GlnGlnThrThrThrLysAlaProThrX^4 IVi (SEQ ID NO:25)

Especially preferred are peptides having a sequence according to one of the figures 2a-i or 5a-d.

The invention further relates to the use of a primer, which contains a component sequence according to one of the formulae Ia-Ih or preferably a sequence according to one of the formulae IIa-IIh or an affiliated complementary sequence, for detecting bacteria of the genus *Listeria*.

The invention also relates to processes for detecting bacteria of the genus *Listeria* by means of a primer which contains a component sequence according to one of the formulae Ia-Ih or preferably a sequence according to one of the formulae IIa-IIh or an affiliated complementary sequence.

The invention further relates to the use of an antibody which is directed against an epitope from the polypeptide sequence according to Figure 3, or which is directed against one of the epitopes having an amino acid sequence according to one of the Figures 2a-i or of the figures 5a-d, for detecting bacteria of the genus *Listeria*.

The invention also relates to processes for detecting bacteria of the genus *Listeria* by means of an antibody which is directed against an epitope from the polypeptide sequence according to Figure 3, or which is directed against one of the epitopes having an amino acid sequence according to one of the Figures 2a-i, or to one of the Figures 5a-d.

The invention finally relates to test kits for detecting bacteria of the genus *Listeria*, in particular of the species *L. monocytogenes*, by means of the amplification of nucleic acids, for example by means of the polymerase chain reaction, which contain a primer having a component sequence according to one of the formulae Ia-Ih or preferably having a sequence according to one of the formulae IIa-IIh, or an affiliated complementary sequence.

The invention furthermore relates to test kits for the immunological detection of bacteria of the species *Listeria monocytogenes*, in which an antibody which is directed against an epitope from the polypeptide sequence according to Figure 3, or which is directed against one of the epitopes having an amino acid sequence according to one of the Figures 2a-i, is contained, as well as to test kits for the immunological detection of bacteria of the species *Listeria innocua*, in which an antibody, which is directed against an epitope having an amino acid sequence according to one of the Figures 5a-d is contained.

Brief Description of the Drawings

Various other objects, features and attendant advantages of the present invention will be more fully appreciated as the same becomes better understood when considered in conjunction with the accompanying drawing, in which like reference characters designate the same or similar parts throughout the several views, and wherein:

Figure 1 shows the result of the electrophoretic separation of amplification products; the experimental details are presented in Example 8.

Figures 2a-i show the amino acid sequences (SEQ ID NO:26-34 respectively) of the particularly preferred immunogenic peptides selected from the sequence of the p60 protein from *Listeria monocytogenes*.

Figure 3 shows the amino acid sequence (SEQ ID NO:39) of the polypeptide selected from the sequence of the p60 protein from *Listeria monocytogenes*, whose epitopes are suitable for the immunological detection of bacteria of the genus *Listeria*.

Figure 4 shows, for comparative purposes, the amino acid sequence (SEQ ID NO:40) of the p60 protein from *Listeria monocytogenes*, which is presented in two component Figures a and b.

Figures 5a-d show the amino acid sequences (SEQ ID NO:35-38 respectively) of the particularly preferred immunogenic peptides selected from the sequence of protein p60 from *Listeria innocua*.

The invention is described below in more detail. In this context, the details of biochemical, immunological and molecular biological processes, which are known to the person skilled in the art and whose details are described in the literature, are presumed. In these processes, use can also be made of variations which are known per se but which are not described in detail here.

The oligonucleotides according to the invention described by the formulae Ia-Ih and IIa-IIh are suitable as primers for nucleic acid amplification methods, and thus for the specific detection of bacteria of the genus *Listeria*. Their sequences are presented in the customary manner, i.e., written from the 5' end to the 3' end. Depending on the requirements of the amplification system being used on any particular occasion, either deoxyribonucleotides or ribonucleotides having the sequences according to the invention are employed. In the latter case, the thymidine moieties are on each occasion replaced by uridine moieties. It is further known to the person skilled in the art that frequently the exchange of one or of a few bases in a nucleic acid sequence does not alter its biological properties. For this reason, the nucleotide sequences according to the invention also comprise those which are derived by base exchange from the sequences Ia-Ih and IIa-IIh, and which biologically show the same effect as the respective primer having the original sequence. Since normally one primer should in each case react with one of the DNA strands, one of the primers is employed in the complementary sequence. The complementary sequence is obtained in a known manner according to the rules for base pairing.

Based on the respective sequence, the oligonucleotides according to the invention can be synthesized by processes known to the person skilled in the art, for example by the phosphotriester or the phosphoamidite method. The phosphoamidite method is preferably employed, in particular using mechanized synthesizers. The method is described in Tetrahedron Lett. (1981) 22:1859-1862. Further details of synthetic processes of this nature are described, for example, in Winnacker, E.L. (1985) Gene und Klone [Genes and Clones], page 44-61 (VCH-Verlagsgesellschaft mbH, Weinheim).

The primers according to the invention are suitable for DNA amplification, for example using the polymerase chain reaction (PCR). For this purpose, the DNA is first dissociated into the single strands by heating. Two primers are used which in each case

hybridize with the homologous DNA segment on one of the DNA strands in each case. The genome segment which lies between these two primers is amplified. The primers attached to the DNA represent the starting points for the amplification. A polymerase, preferably *Taq* DNA polymerase, subsequently completes, in the presence of the four ~~nucleotide~~ triphosphates, the second strand corresponding to the sequence of the original DNA. Subsequently, the double strands which have arisen are dissociated once again into the single strands by heating. This amplification cycle can be repeated a number of times. After a sufficient number of amplification cycles, the amplified nucleic acid can be detected by means of known methods. For this purpose, the DNA can be separated by means of electrophoresis, and subsequently stained with ethidium bromide, and finally detected by fluorescence using UV excitation. Detection using DNA hybridization is also possible. The details of suitable amplification and detection methods are also described in review articles, e.g., Innis et al. (eds.), PCR Protocols (Academic Press, Inc., Harcourt Brace Jovanovich, Publishers). Other nucleic acid amplification processes in which the primers according to the invention can be used are also known from the literature. These include the ligase chain reaction, described by Bond, S. et al. ((1990), pp. 425-434, Raven Press (New York, NY/USA)).

The selection of the primers according to the preferred formulae IIa-IIh determines the position of the start points on the *iap* gene, and thus the specificity of the detection reaction: thus, combinations of primers selected from the sequence of the *iap* gene proved to be unspecific, and consequently unsuitable for detecting listerias by means of DNA amplification (in this connection see, for example, column F in Table 1). However, other selected combinations proved to be specific for the genus *Listeria*, others for groups of *Listeria* species, and others again for individual *Listeria* species. Altogether, therefore, the selection and the composition of the primers is critical. The selection of one of the two primers is always particularly critical, while the second primer can be more easily varied without significantly altering the specificity of the detection reaction. Consequently, according to the teaching of the present invention, for this second primer, a sequence can perfectly well be chosen which does not correspond to one of the formulae Ia-Ih or IIa-IIh.

According to the invention, at least one of the primers is selected from the formulae Ia-Ih or preferably from the formulae IIa-IIh. As already explained, the second primer has substantially less influence than the first primer on the specificity of the amplification reaction. However, combinations are preferred in which both primers are selected from the formulae Ia-Ih or IIa-IIh. Examples of preferred combinations of this nature are (typical results are summarized in Table 1):

a) When using a combination of a primer according to formula IIc with a primer with the complementary sequence according to formula IIb, only the DNA of listerias is amplified, and not the DNA of other types of bacteria (see column D in Table 1).

b) When using a combination of a primer according to formula IId with a primer with the complementary sequence according to formula IIb, only the DNA of *L. monocytogenes* is amplified, and not the DNA of other listerias or other bacteria (see column B in Table 1).

c) When using a combination of a primer according to formula IIf with a primer with the complementary sequence according to formula IIb, only the DNA of particular *Listeria* species is amplified, namely that of *L. seeligeri, L. welshimeri* and *L. ivanovii*, exclusively. This consequently permits group-specific detection (see column E in Table 1).

d) Another example of group-specific detection consists in the use of a combination of a primer according to formula IIh with a primer with the complementary sequence according to formula IIb: only the DNA of *L. grayi* and *L. murrayi* is amplified (see column G in Table 1).

e) Since the amplification products of different *Listeria* species exhibit varying molecular weights, bacteria of the genus *Listeria* can be differentiated by a combination of several primers (according to formulae IId, IIf, IIg and IIh) with the complementary sequence of formula IIb using one single polymerase reaction. Details of this further development of the polymerase technique are evident from Example 8 (see column H in Table 1, as well as Figure 1).

As already mentioned, it is also possible to detect the amplification products by nucleic acid hybridization. To do this, suitable nucleic acid fragments (nucleic acid probes) are added to the reaction mixture after the amplification. These nucleic acid probes possess a base sequence which is completely or partially complementary to the amplified gene segment. In addition, these probes are labelled for a detection reaction: they can contain radioactive isotopes, or carry fluorescent labels, or else be labelled by enzymes. Suitable labelling agents, methods for their introduction into the nucleic acid probe, and detection methods, are known to the person skilled in the art.

In particular, the amplification reaction can be designed specifically for the genus *Listeria* (as described in more detail above under a)) or for a group of *Listeria* species (as described above under c) and d)). By using nucleic acid probes which are in each case specific for one species, the presence of these species of *Listeria* can then be discerned in the reaction mixture. If the probes contain different labelling agents, different

species can also be detected side by side. This variation of the process consequently permits, in a similar manner to that described above under e), the detection of different *Listeria* species side by side.

The use of a nucleic acid probe, or of a mixture of different probes, which react with amplification products of all the *Listeria* species makes it possible to check the specificity of the amplification reaction or to prepare a unitary detection reagent for different *Listeria* species.

The peptides according to the invention, according to formulae IVa-IVi and according to Figure 2a-i or to figure 5a-d, can be incorporated into immunogenic conjugates. Using these conjugates, antibodies can be produced which make it possible specifically to detect bacteria of the genus *Listeria* using immunological methods.

The positions of the peptides according to the invention in the overall sequence of the p60 protein from *Listeria monocytogenes* are given below:

a) The sequence according to formula IIIa begins with proline at position 148 of the p60 sequence (Figure 4a); the peptides according to Figures 2a, 2e and 2f are also located in this region.

b) The sequence according to formula IIIb begins with threonine at position 178 of the p60 sequence (Figure 4a); the peptides according to Figures 2b and 2h are also located in this region.

c) The sequence according to formula IIIc begins with alanine at position 243 of the p60 sequence (Figure 4a); the peptides according to Figures 2c and 2i are also located in this region.

d) The sequence according to formula IIId begins with glutamine at position 292 of the p60 sequence (Figure 4b); the peptide according to Figure 2d is also located in this region.

e) The sequence according to formula IIIe begins with valine at position 71 of the p60 sequence (Figure 4a); the peptide according to Figure 2g is also located in this region.

The sequences of the peptides according to the invention which are shown in Figure 5a-d are derived from the total sequence of protein p60 from *Listeria innocua*; the same holds true for the partial sequences shown in formula IIIf-i and IVf-i.

It is known to the person skilled in the art that the exchange of one or of a few amino acids in a peptide frequently does not alter its biological properties. For this reason, the peptide sequences according to the invention also comprise those which are derived, by amino acid exchange, from the sequences according to Figures 2a-i, according to Figure 5a-d, or according to Figure 3, and which biologically show the same effect

as the respective peptides having the original sequence. One of skill in the art can routinely determine preferred exchanges in accordance with substitutions generally recognized as being preferred, e.g., according to the groups outlined in Dayhoff, M.O., Atlas of Protein Sequence and Structure, Vol. 5, p. 98 (1972), and updates thereof.

The selection of the peptides according to the invention proves to be critical. For example, when a particular peptide,

ThrAsnThrAsnThrAsnThrAsnThrAsnThrAsn

which is encoded by a gene segment around nucleotide 1390 which is specific for *L. monocytogenes*, was selected for the production of antibodies, none of the antisera, surprisingly, showed a reaction with the p60 protein.

Based on the sequence of the amino acids, the peptides can be synthesized by processes which are known to the person skilled in the art, for example by the t_{boc} or by the f_{moc} (tert-butyloxycarbonyl, or 9-fluorenylmethyloxycarbonyl) processes. Details of these processes are described, for example, in J. Am. Chem. Soc. 85, 2149-2154 (1963) and in Synthetic Polypeptides as Antigens (van Regenmortel et al. (eds.), Elsevier 1988 (volume 19 of the series Laboratory Techniques in Biochemistry and Molecular Biology). The f_{moc} process is preferred, in particular mechanized process variations thereof. Details of the process, as well as suitable amino acid protective groups, are known to the person skilled in the art.

Peptides are generally not suitable for producing antibodies. However, if peptides are coupled to high-molecular weight carrier substances, immunogenic conjugates are formed. The peptides according to the invention can be conjugated with known carrier substances. Among these are polyethylene glycols, serum albumins, KLH (keyhole limpet hemocyanin), ovalbumin, glucose dehydrogenase from *Bacillus megaterium* and PPD (purified protein derivative of tuberculin). Preferred carrier substances are KLH and glucose dehydrogenase from *B. megaterium*.

Besides this, bridging compounds (linkers) are frequently employed as well. These are low-molecular weight organic compounds having at least two linkable functional groups. Suitable compounds are known to the person skilled in the art; among these are, for example, 1,2-diaminoethane, succinic acid, β-alanine, 1,6-diaminohexane, 6-aminocaproic acid, adipic acid and cysteine. Cysteine is preferably employed as the linker, with this amino acid residue being incorporated during the synthesis of the peptide. Linkers which contain both an amino and a carboxyl function (e.g., β-alanine, 6-aminocaproic acid or cysteine), can be linked either at the C-terminus or at the N-terminus of the peptide. m-Maleimidobenzoic acid N-hydroxysuccinimide ester

(MBS) is preferably employed for preparing the bonds between the peptide and the carrier substance.

The said immunogenic conjugates serve to produce antibodies in experimental animals according to known processes. Usually, mammals are used for this purpose, for example sheep, goats, rabbits or mice. Rabbits are preferred for producing polyclonal antibodies. However, it is also possible to produce monoclonal antibodies using the immunogenic conjugates according to the invention.(SEQ ID NO:4)

Details of the immunological processes are known to the person skilled in the art. In addition, instructions for carrying out these processes are readily available in the literature; the following may be mentioned by way of example:

- Antibodies, E. Harlow and D. Lane, Cold Spring Harbor (1988)
- Woodard, L.F. and Jasman, R.L. (1985) Vaccine 3, 137-144
- Woodard, L.F. (1989) Laboratory Animal Sci. 39, 222-225
- Handbook of Experimental Immunology, Weir, D.M. et al. eds. (1986): Blackwell Scientific Publications, Oxford, GB.

Among these processes are, for example, the conjugation and immunization processes, as well the preparation and purification of antibodies, and also immunological detection processes. The immunological detection processes in which antibodies according to the invention can be used include preferably agglutination processes, immunometric detection processes, the immunoblot processes and in particular the sandwich ELISA processes.

According to the invention, the concept antibodies embraces both immunoglobulins and antisera. It is furthermore known to the person skilled in the art that, instead of a single antibody which is directed against a single epitope, a mixture of different antibodies of differing specificity may frequently be used. This results in advantages, in particular with regard to the sensitivity of detection. This applies in particular to monoclonal antibodies, but also to other antibodies, which are in each case directed against one epitope. Correspondingly, it can be advantageous to combine a plurality of antibodies which are directed against different peptide structures according to the formulae IIIa-IIIi or according to one of the Figures 3, 2a-i or 5a-d, for the use according to the invention and/or the processes according to the invention.

Details regarding the preparation of the primers and peptides according to the invention, as well as of their use, are evident from the following examples. The person skilled in the art will elicit further methodological details from the cited literature. The examples are intended to illustrate the subject of the invention, and do not represent any limitation of the invention.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius and unless otherwise indicated, all parts and percentages are by weight.

The entire disclosure of all applications, patents and publications, cited above and below, and of corresponding German applications P 42 19 111.4 and P 42 39 567.4, are hereby incorporated by reference.

EXAMPLES

Example 1: Preparation of the primer according to formula IIa

The primer according to formula IIa is prepared by the phosphoamidite method using the 380A DNA synthesizer from Applied Biosystems. The essential features of the method are described in Tetrahedron Lett. (1981) 22:1859-1862. Further details can be found in the literature supplied by the instrument manufacturer.

The primers according to formula IIc, IId, IIf, IIg and IIh are prepared in a corresponding manner. The primers according to formula IIb and IIe are prepared in the respective complementary sequence

(IIb: TTGGCTTCGGTCGCGTAGAATTCATA; (SEQ ID NO:10)

IIe: GCACTTGAATTGCTGTTATTG). (SEQ ID NO:43)

Example 2: Performance of the PCR reaction for the species-specific detection of *L. monocytogenes*

A sample of bacteria containing about 1 μg of DNA, is suspended in 50 μl of buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl) and heated at 110°C for 5 minutes. Subsequently, primers according to formula IId and IIe (see Example 1; in each case 0.4 μg), and 2.5 U of *Taq* polymerase (from Pharmacia), dissolved in reaction buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl), and in each case 200 μmol of dGTP, dATP, dTTP and dCTP are added (total reaction volume 100 μl). The first denaturation step lasts for 3 minutes at 94°C.

Subsequently, the reaction mixture is maintained at a temperature of 55°C for 30 seconds (annealing phase), and at a temperature of 72°C for one minute (elongation phase). The subsequent denaturation steps (at 94°C) last for 45 seconds. After 30 reaction cycles, a concluding elongation step (at 72°C), of 5-minute duration, is carried out.

The PCR products are separated on a polyacrylamide gel (6%) in a running buffer of Tris-borate (in each case 50 mM) containing EDTA (2.5 mM). Subsequently, the separated PCR products are stained with ethidium bromide (0.1 mg/ml in water), and visualized by irradiation with UV light (260 nm).

PCR products are only observed (see column A in Table 1) if DNA or cells from *L. monocytogenes* are present in the sample.

Example 3: Performance of the PCR reaction for the species-specific detection of *L. monocytogenes*

The process described in Example 2 is repeated using primers according to formula IId and IIb (see Example 1) instead of the primers according to formula IId and IIe. In

this case, too. PCR products are only observed if DNA or cells from *L. monocytogenes* are present in the sample (see column B in Table 1).

Table 1: Specificity of the polymerase chain reaction using different primers corresponding to formula IIa-IIh

Combination:	A	B	C	D	E	F	G	H
Primer 1:	IId	IId	IIg	IIc	IIf	IIa	IIh	M[3]
Primer 2:[1]	IIe	IIb	IIb	IIb	IIb	IIb	IIb	IIb
Reaction investigated								
Bacteria:								
L. monocytogenes								
serovar 1/2a EDG	+	+	-	+	-	+	-	+
serovar 1/2a Mack.[2]	+	+	-	+	-	+	-	+
serovar 1/2b	+	+	-	+	-	+	-	+
serovar 1/2c	+	+	-	+	-	+	-	+
serovar 3a	+	+	-	+	-	+	-	+
serovar 3b	+	+	-	+	-	+	-	+
serovar 3c	+	+	-	+	-	+	-	+
serovar 4a	+	+	-	+	-	+	-	+
serovar 4ab	+	+	-	+	-	+	-	+
serovar 4b	+	+	-	+	-	+	-	+
serovar 4c	+	+	-	+	-	+	-	+
serovar 4d	+	+	-	+	-	+	-	+
serovar 4e	+	+	-	+	-	+	-	+
serovar 7	+	+	-	+	-	+	-	+
L. ivanovii	-	-	-	+	+	+	-	+
L. seeligeri	-	-	-	+	+	+	-	+
L. innocua								
serovar 6a	-	-	+	+	-	+	-	+
serovar 6b	-	-	+	+	-	+	-	+
serovar 4ab	-	-	+	+	-	+	-	+
L. welshimeri	-	-	-	+	+	+	-	+
L. murrayi	-	-	-	+	-	+	+	+
L. grayi	-	-	-	+	-	+	+	+
Enterococcus faecalis	-	-	-	-	-	+	-	-
Bacillus cereus	-	-	-	-	-	+	-	-
Micrococcus flavus	-	-	-	-	-	+	-	-

Legend:

- \+ PCR product detected
- \- no PCR product detectable
- 1) Complementary sequence
- 2) Mack.: Mackaness strain
- 3) M: Mixture of primers according to formula IId, IIf, IIg and IIh; Amplification products can be differentiated on the basis of the molecular weight.

Example 4: Performance of the PCR reaction for the genus-specific detection of bacteria of the genus *Listeria*

A sample of bacteria containing about 1 μg of DNA, is suspended in 50 μl water and heated at 110°C for 5 minutes. Subsequently, primers according to formula IIc and IIb (see Example 1; in each case 0.4 μg), and 2.5 U of *Taq* polymerase (from Pharmacia), dissolved in reaction buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl), and in each case 200 μmol of dGTP, dATP, dTTP and dCTP are added (total reaction volume 100 μl). The first denaturation step lasts for 3 minutes at 9°C. Subsequently, the reaction mixture is maintained at a temperature of 56°C for 30 seconds (annealing phase), and at a temperature of 72°C for 2 minutes (elongation phase). The subsequent denaturation steps (at 94°C) each last for 45 seconds. After 30 reaction cycles, a concluding elongation step (at 72°C), of 5-minute duration, is carried out.

The PCR products are separated on an agarose gel (1%) in a running buffer of Tris-borate (in each case 50 mM) containing EDTA (2.5 mM). Subsequently, the separated PCR products are stained by staining with ethidium bromide (0.1 mg/ml in water), and visualized by irradiation with UV light (260 nm).

In this case, PCR products are observed if DNA or cells from bacteria of the genus *Listeria* are present in the sample (see column D in Table 1).

Example 5: Performance of the PCR reaction for the group-specific detection of *listerias*

A sample of bacteria containing about 1 μg of DNA, is suspended in 50 μl water and heated at 110°C for 5 minutes. Subsequently, primers according to formula IIf and IIb (see Example 1; in each case 0.4 μg), and 2.5 U of *Taq* polymerase (from Pharmacia), dissolved in reaction buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl), and in each case 200 μmol of dGTP, dATP, dTTP and dCTP are added (total reaction volume 100 μl). The first denaturation step lasts for 3 minutes at 94°C. Subsequently, the reaction mixture is maintained at a temperature of 58°C for 45 seconds (annealing phase), and at a temperature of 72°C for one minute (elongation phase). The subsequent denaturation steps (at 94°C) last for 45 seconds. After 30 reaction cycles, a concluding elongation step (at 72°C). of 5-minute duration, is carried out.

The PCR products are separated on an agarose gel (1%) in a running buffer of Tris-borate (in each case 50 mM) containing EDTA (2.5 mM). Subsequently, the separated PCR products are stained by staining with ethidium bromide (0.1 mg/ml in water), and visualized by irradiation with UV light (260 nm).

In this case, PCR products are observed only if DNA or cells of bacteria from the group *L. ivanovii, L. seeligeri* and *L. welshimeri* are present in the sample (see column E in Table 1).

<u>Example 6</u>: Performance of the PCR reaction for the species-specific detection of *L. innocua*

A sample of bacteria containing about 1 μg of DNA, is suspended in 50 μl of buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl) and heated at 110°C for 5 minutes. Subsequently, primers according to formula IIg and IIb (see Example 1; in each case 0.4 μg), and 2.5 U of *Taq* polymerase (from Pharmacia), dissolved in reaction buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl), and in each case 200 μmol of dGTP, dATP, dTTP and dCTP are added (total reaction volume 100 μl). The first denaturation step lasts for 3 minutes at 94°C. Subsequently, the reaction mixture is maintained at a temperature of 62°C for 60 seconds (annealing phase), and at a temperature of 72°C for 45 seconds (elongation phase). The subsequent denaturation steps (at 94°C) last for 45 seconds. After 30 reaction cycles, a concluding elongation step (at 72°C), of 5-minute duration, is carried out.

The PCR products are separated on an agarose gel (1%) in a running buffer of Tris-borate (in each case 50 mM) containing EDTA (2.5 mM). Subsequently, the separated PCR products are stained by staining with ethidium bromide (0.1 mg/ml in water), and visualized by irradiation with UV light (260 nm).

PCR products are only observed if DNA or cells of *L. innocua* are present in the sample (see column C in Table 1).

<u>Example 7</u>: Performance of the PCR reaction for the group-specific detection of *listerias*

A sample of bacteria containing about 1 μg of DNA, is suspended in 50 μl of water and heated at 110°C for 5 minutes. Subsequently, primers according to formula IIh and IIb (see Example 1; in each case 0.4 μg), and 2.5 U of *Taq* polymerase (from Pharmacia), dissolved in reaction buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl), and in each case 200 μmol of dGTP, dATP, dTTP and dCTP, are added (total reaction volume 100 μl). The first denaturation step lasts for 3 minutes at 94°C. Subsequently, the reaction mixture is maintained at a temperature of 56°C for 45 seconds (annealing phase), and at a temperature of 72°C for 45 seconds (elongation phase). The subsequent denaturation steps (at 94°C) last for 45 seconds. After 30 reaction cycles, a concluding elongation step (at 72°C), of 5-minute duration, is carried out.

The PCR products are separated on an agarose gel (1%) in a running buffer of Tris-borate (in each case 50 mM) containing EDTA (2.5 mM). Subsequently, the separated PCR products are stained by staining with ethidium bromide (0.1 mg/ml in water) and visualized by irradiation with UV light (260 nm).

In this case, PCR products are only observed if DNA or cells of bacteria from the group *L. grayi* and *L. murrayi* are present in the sample (see column G in Table 1).

<u>Example 8</u>: Performance of a combined PCR reaction for the species-specific detection of *L. monocytogenes* and of *L. innocua* and for the group-specific detection of the groups *L. ivanovii/L. seeligeri/L. welshimeri* and *L. grayi/L. murrayi*

A sample of bacteria containing about 1 μg of DNA, is suspended in 50 μl of buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl) and heated at 110°C for 5 minutes. Subsequently, a mixture of primers according to formula IId, IIf, IIg, IIh and IIb (see Example 1; in each case 0.4 μg), as well as 2.5 U of *Taq* polymerase (from Pharmacia), dissolved in reaction buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl), and in each case 200 μmol of dGTP, dATP, dTTP and dCTP, are added (total reaction volume 100 μl). The first denaturation step lasts for 3 minutes at 94°C. Subsequently, the reaction mixture is maintained at a temperature of 56°C for 45 seconds (annealing phase), and at a temperature of 72°C for one minute (elongation phase). The subsequent denaturation steps (at 94°C) last for 45 seconds. After 30 reaction cycles, a concluding elongation step (at 72°C), of 5-minute duration, is carried out.

The PCR products are separated on a polyacrylamide gel (4%) in a running buffer of Tris-borate (in each case 50 mM) containing EDTA (2.5 mM). In addition, a nucleic acid mixture (for example the product resulting from cleavage of *Spp*1 phage DNA by the restriction endonuclease *Eco*RI) is included as a molecular weight standard. Subsequently, the separated PCR products are stained by staining with ethidium bromide (0.1 mg/ml in water), and visualized by irradiation with UV light (260 nm).

The presence of DNA or cells of bacteria from the species *L. monocytogenes*, from the species *L. innocua*, from the group *L. ivanovii/L. seeligeri/L. welshimeri* or from the group *L. grayi/L. murrayi* can be differentiated on the basis of the different molecular weights (see column H in Table 1, as well as Figure 1).

<u>Example 9</u>: Synthesis of the peptide

CysGlnGlnGlnThrAlaProLysAlaProThrGlu (SEQ ID NO:42)

The f_{moc} process (9-fluorenylmethyloxycarbonyl protective group) is used for the synthesis of the peptide CysGlnGlnGlnThrAlaProLysAlaProThrGlu. (SEQ ID NO:42) This peptide cor-

responds to a peptide of the formula IVd with an additional N-terminal cysteine residue as linker. A peptide synthesizer from Applied Biosystems is used for the synthesis; the process parameters are contained in the instrument documentation.

A polymeric support with 4-(2'4'-dimethoxyphenylaminomethyl)phenoxy groups serves as the solid phase. The amino acids are employed as α-N-f_{moc} derivatives. Any reactive side groups contained in the amino acids are masked by additional protective groups which may be eliminated by hydrolysis with trifluoroacetic acid. The peptide bonds are produced by activating the carboxyl groups with diisopropylcarbodiimide. The order in which the amino acid derivatives are put in is determined by the desired sequence.

In the first step of the synthesis cycle, the amino group on the solid phase, i.e., in the first cycle the amino groups of the 4-(2'4'-dimethoxyphenylaminomethyl)phenoxy residue of the support, reacts with the carboxyl group of the incoming amino acid, which is employed as the α-N-f_{moc} derivative, where appropriate with protected side chains, and which is activated by diisopropylcarbodiimide, as does the α-amino group of the last amino acid to be attached in the following cycles. Amino acid derivatives which have not reacted are washed out with dimethylformamide. Subsequently, the f_{moc} group is eliminated by treating with 20% (V/V) piperidine in dimethylformamide. The rest of the protective groups remain unaltered during this reaction. Following the removal of the α-N-protective group, the next reaction cycle can begin. Once the last amino acid corresponding to the envisaged sequence has been added, the protective groups of the side chains and the bond with the support resin are cleaved by acid hydrolysis with trifluoroacetic acid. The peptide is subsequently purified by high pressure liquid chromatography.

The remaining peptides with the sequences according to the invention are also synthesized in accordance with the procedure described above.

__Example 10__: **Conjugation of the peptide CysGlnGlnGlnThrAlaProLysAlaProThrGlu with glucose dehydrogenase** (SEQ ID NO:42)

__a) Derivatization of the glucose dehydrogenase__: 30 mg of glucose dehydrogenase from *Bacillus megaterium* from Merck (Art. No. 13732) are dissolved in 4 ml of sodium phosphate buffer (50 mM; pH 8.0). 6.78 mg of N-y-maleimidobutyryloxysuccinimide (from Calbiochem), dissolved in 50 μl of dimethyl sulfoxide, are added to 2.4 ml of this solution, and the mixture is left to stand at room temperature for 30 minutes. Subsequently, the excess N-y-maleimidobutyryloxysuccinimide is separated off chromatographically by gel filtration on PD-10 (from Pharmacia). Following the chromatography,

3.5 ml of a solution of the activated carrier protein are obtained, having a concentration of 4.5 mg/ml.

b) Coupling with the peptide: 5.2 mg of the peptide, prepared according to Example 9 and dissolved in 1 ml of sodium phosphate buffer (50 mM; pH 7.0), are added to 1.1 ml of the solution from the above step and the mixture is left to stand at room temperature for 3 hours. Subsequently, the peptide which has not been bound is separated off chromatographically by gel filtration on PD-10 (from Pharmacia). Following the chromatography, 3.5 ml of a solution of the conjugate are obtained, having a concentration of 2.3 mg/ml.

Conjugates with other peptides corresponding to the present invention are also prepared in accordance with the procedure described above.

Example 11: Production of polyclonal antibodies against the peptide CysGlnGlnGlnThrAlaProLysAlaProThrGlu (SEQ ID NO:42)

Two rabbits are in each case injected intramuscularly with an emulsion consisting of 0.18 ml of conjugate from Example 10, 0.07 ml of phosphate-buffered saline and 0.25 ml of an oil adjuvant (MISA 50, from Seppic, France). Booster injections of the same quantities are given three, five and seven weeks after the initial injection. One week after the last injection, the animals are killed and exsanguinated. After the blood has coagulated, the antiserum is obtained by centrifugation and sodium azide is added to give a final concentration of 0.02%. The antiserum is stored frozen at -20°C.

Example 12: Production of monoclonal antibodies against the peptide CysGlnGlnGlnThrAlaProLysAlaProThrGlu (SEQ ID NO:42)

Two mice are in each case injected subcutaneously with an emulsion consisting of 0.1 ml of conjugate from Example 10 and 0.1 ml oil adjuvant (MISA 50, from Seppic, France). Booster injections of the same amounts are given two, four and six weeks after the initial injection. Three days after the last injection, the animals are killed and the spleen is isolated. The cells from the spleen are isolated by customary processes and fused with a permanent murine cell line. Cell lines which form antibodies ~~again~~ against the peptide CysGlnGlnGlnThrAlaProLysAlaProThrGlu (SEQ ID NO:42) are selected from the fusion products.

Example 13: Immunological detection of *L. monocytogenes*

a) Pre-culture and centrifugation of the bacteria: 10 ml of CASO broth are inoculated with material from several colonies of *L. monocytogenes* and incubated at 30°C

overnight. Subsequently, 1 ml of the culture is withdrawn in each case. The bacterial cells are removed by centrifugation (13000 rpm).

<u>b) Identification reaction</u>: In each case 300 μl of the supernatants from the previous step are pipetted into the wells of a microtiter plate and incubated at 4°C overnight. Subsequently, each well is washed three times with 100 μl of washing solution (9 g/l NaCl and 0.05% Tween 20 in water) on each occasion. 100 μl of antiserum prepared according to Example 11 are now pipetted into each well and the plate is incubated at room temperature for one hour. Each well is once again washed three times with 100 μl of washing solution on each occasion. 100 μl of anti-rabbit antibody solution labelled with alkaline phosphatase (Art. No. A 8025, from Sigma) are then pipetted into each of the wells, and the plate is incubated at room temperature for 30 minutes. Each of the wells is washed once again with 100 μl of washing solution and the bound, enzyme-labelled antibody is subsequently detected. To do this, 200 μl of a buffer solution containing substrate is added to each well, and the plate is incubated at room temperature for 30 minutes. The reaction is stopped by the addition of 100 μl of 2N NaOH solution (Art. No. 9136, from Merck) to each well, and the reaction product is rendered visible. A yellow-orange coloration indicates the presence of *L. monocytogenes*.

<u>Example 14</u>: Specific detection of *L. monocytogenes* using immunoblotting

Bacteria are pre-cultured as described in Example 13a), and the cells are centrifuged off. The cells which have been centrifuged off are taken up and suspended in 1 ml of phosphate-buffered saline. 2 μl of this suspension are pipetted on to a nitrocellulose membrane (Hybond C, 0.45 μm, Art. No. RPN 283 C, from Amersham). Once the solution has dried in, the membrane is treated at room temperature for one hour with a solution of bovine serum albumin (10 g/l) in phosphate-buffered saline. A dilution (1:200) of the antiserum obtained in Example 11 is prepared using a solution of bovine serum albumin (10 g/l) and Tween 20 (0.5 g/l) in phosphate-buffered saline (antibody solution A), as is a further dilution (1:500) of peroxidase-labelled anti-rabbit antibody (anti-rabbit IgG, Art. No. 68-397; from ICN Immunobiologicals) using the same diluent (HRP-antibody solution). The membrane is incubated at room temperature for one hour with antibody solution A. and subsequently washed three times with phosphate-buffered saline containing 0.05% Tween 20. In order to detect the antibody binding, the membrane is subsequently incubated at room temperature for one hour with HRP-antibody solution and in each case washed three times with a) Tween 20 (0.5 g/l) in phosphate-buffered saline, b) phosphate-buffered saline and c) Tris buffer (50 mM; pH 7.4; containing 200 mM NaCl). For the color reaction, a solution of 4-chloro-1-naphthol

(3 mg/ml in methanol) is diluted with five volumes of Tris buffer (50 mM; pH 7.4; containing 200 mM NaCl), and hydrogen peroxide is added (final concentration 0.1 g/l). The membrane is incubated in this substrate solution. A blue-black coloration indicates *L. monocytogenes*.

The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.